SUICIDE EXPRESSION VECTOR FOR VACCINE STRAINS

Field of the Invention:

The present invention relates to suicide expression vectors particularly for use in the production of microorganism vectors intended for environmental release. In a particular application of the invention, the expression vector is used in the production of a bacterial vaccine bait for sterilisation of vermin species.

Background of the Invention:

There is a worldwide concern regarding the environmental release of genetically engineered microorganisms (GEMs) (Wilson & Lindow, 1993). Microorganisms harbour efficient mechanisms for horizontal gene transfer that enable them to adapt to environmental changes. Conjugation, transduction, transformation and retromobilization are the main mechanisms that contribute to the flux of genes within microbial communities (Veal *et al.*, 1992).

In recent times, conditional suicide systems have been developed to address this problem, particularly for use in GEMs involved in bioremediation of contaminated soils and in waste treatment. These systems however, involve the use of inducible lytic systems which lyse the bacterial cell (e.g. Bej *et. al.*, 1988). Such systems would not be applicable to live recombinant vaccines that use bacterial delivery vectors since they would lyse the bacterial cells prior to vaccination. For such vaccines to be successful in eliciting antigen-specific immune responses, they must be live. Early work has demonstrated that killed bacterial vectors are not as effective as live ones. Such recombinant vaccines if used in baits could pose the hazard of transferring the recombinant DNA to other organisms in the environment.

In order to overcome this problem, the present inventors have developed a suicide expression vector system which results in the selective degradation of the recombinant expression vector DNA without destroying chromosomal DNA, prior to release of the microorganism vector into the environment.

Disclosure of the invention:

Thus, in a first aspect, the present invention provides a suicide expression vector for expressing a heterologous peptide, polypeptide or protein in a selected host cell, said vector comprising:

(i) a first nucleotide sequence encoding said heterologous peptide, polypeptide or protein operably linked to a first promoter sequence,

(ii) a second nucleotide sequence encoding a restriction enzyme or functional portion thereof operably linked to a second promoter sequence, said second promoter sequence being inducible, and

(iii) one or more cleavage site(s) for said restriction enzyme or functional portion thereof, said cleavage site(s) being absent from the chromosomal DNA of said host cell,

wherein upon introduction of the vector into said host cell, induced expression of the restriction enzyme or functional portion thereof from said second nucleotide sequence brings about the cleavage of the suicide expression vector.

The suicide expression vector according to the present invention may be transformed into a host cell, preferably a bacterial or yeast host cell, and used to produce a desired heterologous peptide, polypeptide or protein. Once sufficient expression of the heterologous peptide, polypeptide or protein has occurred, the transformed host cell may be induced to express the restriction enzyme or functional portion thereof thereby causing the cleavage and subsequent degradation of the expression vector.

The host cell and the restriction enzyme/cleavage site(s) are selected so as to ensure that the expression of the restriction enzyme brings about the cleavage of the recombinant expression vector only. In other words, the host cell and restriction enzyme/cleavage site(s) are selected so as to ensure that the host's DNA is not cleaved by the expressed restriction enzyme. Selection may be readily made by isolating host DNA from a test microorganism by known methods, subjecting the isolated host DNA to a candidate restriction enzyme under suitable conditions, and analysing the host DNA by, for example, gel electrophoresis for any cleavage. If no cleavage has occurred then this should indicate that the host DNA does not include a cleavage site for the candidate restriction enzyme. Preferably, the restriction enzyme is selected from those that recognise cleavage sites of ten or more nucleotides such as I-*Ppo*I, I-*Ceu*I, P1-*Psp*I, P1-*T1i*I and P1-*Sce*I.

The first promoter sequence may be a constitutive promoter sequence but, more preferably, is an inducible promoter. However, the second promoter sequence must, as stated above, be an inducible promoter. Moreover, it is an important feature of the present invention that the second promoter sequence is not excessively "leaky" prior to induction, since premature expression of the restriction enzyme or functional portion thereof may lead to the cleavage and subsequent degradation of the expression vector.

Induction of the second promoter sequence may be achieved by providing an inducer molecule that interacts with a protein which represses transcription. Examples of a promoter able to be induced in this manner are the p*lacZ* promoter, the p*lacUV5* promoter and the T7 RNA polymerase promoter. Other methods for inducing the inducible second promoter sequence may be through exposure to UV radiation or heat shock or environmental stress such as modulation of concentration of nutrients, oxygen, pH etc. However, preferably, the inducible second promoter sequence is induced through the expression of a peptide, polypeptide or protein required to initiate or otherwise cause transcription from the second promoter sequence. In this manner of induction, expression of the peptide, polypeptide or protein required for transcription, is placed under the control of an inducible promoter such as those mentioned above.

Thus, in preferred embodiments of the invention, the second promoter sequence is a promoter sequence which is unrecognised by the RNA polymerase(s) of the host cell, and the expression vector further comprises an additional nucleotide sequence encoding an RNA polymerase for the second promoter sequence operably linked to an inducible promoter.

In a particularly preferred embodiment of the invention, the second promoter sequence is a T7 RNA polymerase promoter sequence which is unrecognised by the RNA polymerase of the host cell and the expression vector further comprises a nucleotide sequence encoding T7 RNA polymerase operably linked to an inducible promoter sequence such as the *lacUV5* promoter which is induced by isopropyl-β-thiogalactopyranoside (IPTG) (alternatively, the host cell includes on its chromosome(s) or on a plasmid(s) a nucleotide sequence encoding T7 RNA polymerase operably linked to an inducible promoter sequence such as the p*lacUV5* promoter). However, since the p*lac*UV5 promoter is prone to "leakage", the expression

vector of this preferred embodiment may also comprise a nucleotide sequence encoding lysozyme expressibly linked to a constitutive promoter sequence. Constitutive expression of the lysozyme will inhibit the "leaky" expression of T7 RNA polymerase and thereby prevents the premature expression of the restriction enzyme or functional portion thereof.

As a "safeguard" against premature expression of the restriction enzyme or functional portion thereof, the expression vector preferably further comprises a nucleotide sequence encoding a ribozyme targetted against the mRNA produced from the nucleotide sequence encoding the restriction enzyme or functional portion thereof. The ribozyme encoding nucleotide sequence is operably linked to a constitutive or inducible promoter sequence. The ribozyme should be expressed such that it will be present to immediately cleave low "leakage" amounts of mRNA encoding the restriction enzyme or functional portion thereof. Induced expression of the restriction enzyme or functional portion thereof will overwhelm the cleavage activity of the ribozyme and thus result in the cleavage and subsequent degradation of the expression vector.

In a second aspect, the present invention provides a host cell transformed with a suicide expression vector according to the first aspect.

In a third aspect. the present invention provides a method of expressing a heterologous peptide, polypeptide or protein in a selected host cell, comprising;

(i) transforming said host cell with a suicide expression vector according to the first aspect,

(ii) culturing said transformed host cell under suitable conditions for the expression of the said heterologous peptide, polypeptide or protein, and

(iii) thereafter inducing expression of the restriction enzyme or functional portion thereof to bring about cleavage of the said suicide expression vector.

In a fourth aspect, the present invention provides a method for the production of a microorganism vector which contains recombinant peptide, polypeptide or protein but no recombinant DNA, comprising:
(i) transforming said microorganism with a suicide expression vector according to the first aspect,
(ii) culturing said transformed microorganism under suitable conditions for the expression of the said heterologous peptide, polypeptide or protein, and
(iii) thereafter inducing expression of the restriction enzyme or functional portion thereof to bring about cleavage of the said suicide expression vector.

Preferably, the microorganism vector is intended for enviromental release in the applications of, for example, vaccine baits for sterilisation of vermin, vaccination against animal pathogens, compositions for bioremediation of contaminated soils and waste, and insecticidal compositions for crop spraying. In such applications, the heterologous peptide, polypeptide or protein contained in, or expressed on the surface of the microorganism may be, respectively, a zona pellucida or sperm or hormone antigen, bacterial, viral or parasite antigen, an esterase capable of hydrolysing organophosphates, and an insecticidal toxin such as Bt toxin.

In a fifth aspect, the present invention provides a microorganism vector produced by the method according to the fourth aspect.

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

The invention will hereinafter be described by way of the following non-limiting examples and accompanying figures.

Brief description of the accompanying figures:

FIGURE 1. PLASMID CONSTRUCTION.

The genetic construction of the various plasmids used in this study is shown. Only the relevant segments of each plasmid are shown. The oligonucleotides (RBS numbers) used for each construct are shown in italics. Plasmid names are shown in a box. The abbreviations used include: MCS, multiple cloning site; p*aer*, iron regulated aerobactin promoter; AmpR,

ampicillin resistance gene; p*T7*, phage T7 promoter; p*T3*, phage T3 promoter; p*OmpA*, *E. coli* outer membrane A promoter; p*lac*, *E. coli* lactose operon promoter; *lac*O, *E. coli* lactose operon operator; *lac*I, *E. coli* lactose operon repressor.

FIGURE 2. DEMONSTRATION OF FUNCTION OF I-*Ppo*I IN *E. coli*.

The plasmids pRBS 22 and pI3-941 were transformed into JM 109 (DE3) and examined for self-restriction upon induction with IPTG. Two isolates of pI3-941 and one of pRBS22 in JM109 (DE3) were grown to OD_{600} = 0.5, induced with 1mM IPTG for 1 hour and plasmid prepared by the alkaline lysis method. Plasmid profiles of uninduced and induced cultures are shown. The recombinant plasmid is indicated with an arrow. The molecular weight marker used is λ *Hind*III.

FIGURE 3. SYNTHETIC RIBOZYME CONSTRUCT.

The oligonucleotides RBS 67a, 68, 69, 70, 71, 72 were used to construct the ribozyme genetic cassette. Ribozyme expression is under the control of *ompA* promoter and transcription is terminated at the T7 terminator. The ribozyme targets bases 345-369 of the I-*Ppo*I intron 3 sequence (Muscarella et al., 1990). Restriction sites and other relevant regions are shown below the DNA sequence.

FIGURE 4. I-*Ppo*I ACTIVITY CONTROLLED WITH RIBOZYME.

Four different isolates of pRBS 43 were transformed into JM109 (DE3) and examined for plasmid maintenance in the uninduced state and for self-restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. Plasmid profiles from uninduced and induced cells are shown. The molecular weight marker used is λ *Hind* III and the plasmid band is indicated with an arrow.

FIGURE 5. EFFECT OF GROWTH TEMPERATURE ON PLASMID SELF-RESTRICTION.

Three independent isolates of recombinant JM109 (DE3) strain carrying plasmid pRBS 45 were grown to OD_{600} = 0.5 either at 37°C or 42°C and all cultures were induced at 37°C with 1mM IPTG for 1 hour. Plasmid profiles

for each culture are shown. The plasmid is indicated with an arrow. λ *Hind* III molecular weight marker is also shown.

FIGURE 6. EFFECT OF THE IRON-REGULATED PROMOTER (*paer*) ON PLASMID SELF-RESTRICTION.

Six different isolates of pRBS 46 were transformed into JM109 (DE3) and examined for self restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. Plasmid profiles of uninduced and induced cultures are shown. The molecular weight marker is λ *Hind* III and the plasmid band is indicated with an arrow.

FIGURE 7. EFFECT OF THE IRON-REGULATED PROMOTER (WITHOUT UP-STREAM PROMOTERS) ON PLASMID SELF-RESTRICTION.

Six different isolates of pRBS 47 were transformed into JM109 (DE3) and examined for self-restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. Plasmid profiles from uninduced and induced cells are shown. The molecular weight marker λ *Hind* III is shown and the plasmid band is indicated with an arrow.

FIGURE 8. DEMONSTRATION OF EFFECTIVE SUICIDE PLASMID.

Six different isolates of pRBS 48 were transformed into JM109 (DE3) and examined for self-restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. The molecular weight marker λ *Hind* III is shown and the plasmid band is indicated with an arrow.

FIGURES 9A TO 9F. OVERVIEW OF THE BASIC CONCEPT OF THE SUICIDE PLASMID FOR VACCINE AND/OR BIOREMEDIATION STRAINS.

The complete concept is outlined in the figures 9A to 9F which show the sequential steps involved in initially expressing large quantities of the recombinant protein followed by elimination of all recombinant DNA.

TABLE 1. LIST OF OLIGONUCLEOTIDES.

The DNA sequence and relevant details for each of the synthetic oligonucleotides used in this study are shown.

OLIGONUCLEOTIDE	DNA SEQUENCE
RBS 3 (SEQ ID NO. 1)	5' Spacer: AGCT SmaI: CCCGGG I-PpoI from 5' end: CTCTCTTAAGGTAG SmaI: CCCGGG Spacer: AGCT 3'
RBS 4 (SEQ ID NO. 2)	5' Spacer: AGCT SmaI: CCCGGG I-PpoI from 3' end: CTACCTTAAGAGAG SmaI: CCCGGG Spacer: AGCT 3'
RBS 67a (SEQ ID NO. 3)	5' Spacer: GAATC XhoI: CTCGAG OmpA promoter start: GAGTTCACATTGTAAGTTTTC 3'
RBS 68 (SEQ ID NO. 4)	5' Spacer: AAGAG BglII: AGATCT OmpA promoter from 3' end: AGTCTACAACGTAGTTGAAAACTTACAATGTGAACTCC 3'
RBS 69 (SEQ ID NO. 5)	5' Spacer: AGACT BglII: AGAT I-PpoI from 5' end: CTCTCTTAAGGTAGC Region 369-358 of I-PpoI gene: TTGTCGTCTAGT Catalytic domain of Ribozyme from 5' end: CTGATGAGTCCG 3'
RBS 70 (SEQ ID NO. 6)	5' AGGGGTTATGTGTGCTGGGAGTTTCGTCCTCACGGACTCATCAGACTA 3'
RBS 71 (SEQ ID NO. 7)	5' AGCACACATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTG 3'
RBS 72 (SEQ ID NO. 8)	5' GACTTGTCGACAAAAACCCCTCAAGACCCG 3'
RBS 87 (SEQ ID NO. 9)	5' Spacer: GATCAT XhoI: CTCGAG 5' end of aerobactin promoter: CGCCATATCCTCCCAGAG 3'
RBS 88 (SEQ ID NO. 10)	5' Spacer: GATCAT BglII: AGATCT 3' end of aerobactin promoter: ACACAGTAAAATAATAAC 3'

Design for a suicide expression vector according to the invention:

A design for a preferred suicide expression vector according to the invention is shown diagrammatically in Fig. 9A TO 9F. The expression vector includes the following elements:

(i) foreign ("vaccine") antigen expressing gene cassette (including a constitutive or inducible promoter sequence),

(ii) antibiotic or other resistance or selection marker,

(iii) origin of replication,

(iv) restriction enzyme gene (e.g.. I-*Ppo*I) with an inducible gene expression promoter (e.g. T7 promoter sequence),

(v) ribozyme catalytic region,

(vi) an inducible promoter to express the ribozyme (e.g. p*aer* promoter),

(vii) a transcription terminator to terminate the mRNA transcript originating from the promoter mentioned in (vi), and

(viii) one or more cloned intron-encoded restriction enzyme (e.g. I-*Ppo*I) cleavage sites.

EXAMPLE 1:- Bacterial vaccine transformed with a suicide expression vector.

A recombinant suicide expression vector of the above design, carrying gene(s) encoding vaccine antigen(s), under the control of an *in vitro* inducible promoter is transformed into a bacterial vaccine delivery vector such as *Salmonella typhimurium aro*A- by any of the methods well known to the art. The recombinant *S. typhimurium aro*A- strain is induced to activate the promoter and express high levels of the vaccine antigen(s). Prior studies on the kinetics of vaccine antigen expression in the recombinant *Salmonella* would indicate the time required to achieve maximal expression of the vaccine antigen(s). The recombinant *Salmonella* would therefore be induced for the length of time required for maximal vaccine antigen expression. The growth media may also include an inducer for the promoter sequence controlling expression of a ribozyme catalytic region (e.g. the growth media would include 2',2-dipyridyl (an iron chelator) to induce expression of ribozyme from a ribozyme catalytic region under the control of the *paer* promoter. Addition of IPTG (isopropyl-β-thiogalactopyranoside) to the growth media would induce the p*lacUV5* controlled expression of the T7 RNA

polymerase protein from a T7 RNA polymerase gene preferably present on the suicide expression vector. Induced expression of the T7 RNA polymerase would be expected to overcome the repressing effect of the ribozyme on T7 promoter driven expression of I-*Ppo*I restriction enzyme and thereby lead to the expression of I-*Ppo*I restriction enzyme to effect cleavage of the suicide expression vector. Additionally, it may be possible to repress *paer* promoter with the addition of $FeCl_3$ into the growth media. Such repression may decrease expression of the ribozyme to basal levels permitting greater I-*Ppo*I activity. The *Salmonella typhimurium aro*A- chromosome does not carry any I-*Ppo*I sites. Once cleaved, the bacterial endogenous exonucleases would be expected to degrade the resulting linear fragments of plasmid DNA.

The kinetics of the loss of the suicide expression vector from the bacterial cells can be analysed to determine the time required for complete loss of expression vector DNA from the vaccine preparation. Simultaneous analyses can also be carried out to ensure that the vaccine antigen in the absence of any further expression following addition of IPTG is sufficient to elicit an effective immune response.

The vaccine strain carrying maximal amounts of the vaccine antigen but completely devoid of recombinant expression vector DNA would then be ready for release into the environment e.g. in baits.

EXAMPLE 2:- Expression of restriction enzyme I-*Ppo*I.

Materials and methods:

Materials

Plasmid pI3-941 (Muscarella *et. al.*, 1990) with the gene for the restriction enzyme I-*Ppo*I was provided by the laboratory of Dr Vogt. Expression vector pET 21d was obtained from Novagen (Madison. WI., U.S.A.), general molecular biology reagents were sourced from Boehringer Mannheim, Promega and New England Biolabs and general laboratory reagents were from Sigma. *E. coli* strain JM109 (DE3) (Yanisch-Perron *et. al.*, 1985) was purchased from Promega Corp., (Madison. WI., U.S.A.) and was used for the transformation of plasmid constructs. The strain carries chromosomally integrated bacteriophage lambda which carries the gene encoding T7 RNA polymerase. *Salmonella typhimurium aro*A- strain H4335 (Brahmbhatt *et. al.*, 1997) was used as a vaccine carrier strain.

Synthetic oligonucleotides used in PCR reactions were synthesised by Ransom Hill Bioscience Inc., (Ramona, CA., U.S.A.) and are listed in Table1. *Pfu* turbo was obtained from Stratagene.

Methods

All DNA manipulation experiments were performed as standard molecular biology techniques (Sambrook *et.al.*, 1989).

Expression of I-*Ppo*I from the plasmid constructs was determined by growing the bacterial cells harbouring the expression plasmid to $0D_{600} = 0.5$ and inducing with 1 mM IPTG for 1 to 3 hours.

Genetic construction of ribozyme (Figure 3):

The oligos RBS 68, 69, 70 and 71 (Table 1) were pooled in equal amounts and diluted to give a final concentration of 50 ng/µl of DNA. One microlitre of this pooled DNA was used as the template for PCR. The oligos RBS 67a and 72 (Table 1) were used as the PCR primers. The reaction consisted of 0.3 mM each dNTP, 0.8 µM of each PCR primer, 50 ng of template DNA, 2.5 units of *pfu* turbo DNA polymerase and 4 mM $MgSO_4$ all in 50 µl of reaction buffer. The annealing temperature was 65°C for one minute,extension at 72°C was for 30 seconds and denaturation at 94°C for 1min. The PCR product was precipitated and digested with*Xho* I and *Sal* I and cloned into the vector pI3-941 prepared with the same enzymes. The resulting vector was called pRBS 43 and was confirmed by digestion with*Xho* I and *Sal* I together and with *Bgl* II alone.

Results:

Demonstration that I-*Ppo*I does not cleave *E. coli* & *S. typhimurium* chromosomal DNA.

Chromosomal DNA was prepared from a variety of *E. coli* and *Salmonella* strains and incubated *in vitro* with purchased enzyme I-*Ppo*I. The same DNAs were also incubated with other restriction enzymes e.g. *Eco*R I, *Not* I, *Xba* I and *Hind* III. All incubations were carried out in the provided incubation buffer. The DNAs were analysed by agarose gel electrophoresis and ethidium bromide staining (data not shown). The results clearly showed that there were no cleavage sites for I-*Ppo*I in the *E. coli* and *Salmonella* chromosomes. This is significant since if the chromosome contains the I-*Ppo*I site then the concept of suicide plasmid would not be applicable since

cytoplasmic expression of I-*Ppo*I would be deleterious to the viability of the carrier strain to be used as a vaccine or for environmental bioremediation.

Plasmid Construction and analysis of I-*Ppo*I function in recombinant *E. coli*.

Plasmid pI3-941 (Fig. 1) carries the I-*Ppo*I restriction enzyme encoding gene and the enzyme is expressed under the control of the T7 promoter. As a first step, it was necessary to determine the following:

(1) Is the eucaryotic I-*Ppo*I restriction enzyme functional in procaryotic Gram-negative bacteria such as *E. coli*.

(2) If the I-*Ppo*I enzyme does cleave plasmid DNA carrying the I-*Ppo*I recognition site, then do the endogenous *E. coli* exo- and/or endo-nucleases digest the recombinant plasmid.

(3) If (1) and (2) are achieved, then would the recombinant plasmid cleavage be sufficient to completely eliminate recombinant DNA from the bacterial cell.

To answer the above questions the following set of recombinant plasmids were constructed and analysed for self-restriction and cellular plasmid maintenance in the uninduced and induced (for I-*Ppo*I expression) state.

The I-*Ppo*I restriction enzyme cleavage site was initially cloned into plasmid pI3-941 to give plasmid pRBS 22. To accomplish this, plasmid pI3-941 was cleaved with *Xho* I, end-filled with T4 DNA polymerase, phosphatased and ligated to annealed, *Sma* I cleaved oligos RBS 3 and RBS 4. The resulting plasmid pRBS 22 (Fig. 1) carries the I-*Ppo*I restriction enzyme site harboured in the synthetic oligonucleotide insert DNA.

Both plasmids (pI3-941 and pRBS 22) were transformed into the host strain JM109 (DE3) for analysis of expression of the I-*Ppo*I enzyme from the T7 promoter. Agarose gel analysis of plasmid profiles from the recombinant strains revealed (Fig. 2) that even in the uninduced state the plasmid pRBS 22 was eliminated to such low levels that it was not readily visible by ethidium bromide staining. This data indicated that the eucaryotic I-*Ppo*I restriction enzyme is functional in the cytoplasm of procaryotic *E. coli*. The enzyme activity is very high and hence even residual (leaky) expression of I-*Ppo*I from the T7 promoter is sufficient to cleave the recombinant plasmid. The data also demonstrates that once I-*Ppo*I cleavage occurs, the *E. coli* endogenous nucleases are able to completely eliminate the recombinant plasmid.

The data indicates that stringent repression of the promoter is desirable to maintain the plasmid in the cell in the uninduced state which is required to ensure that recombinant DNA gene product(s) are expressed to high levels before elimination of recombinant DNA. Thus, with a desire to fully repress promoter expression in the uninduced plasmid, it was hypothesised that the use of a ribozyme (anti-sense catalytic RNA), expressed at low to medium levels, that targets the I-*Ppo*I mRNA, may reduce enzyme expression to a level where the plasmid is stable but still allows self-restriction upon high level induction of expression of I-*Ppo*I.

A synthetic DNA sequence was designed (Fig. 3, SEQ ID NO. 11) which carries the ribozyme catalytic DNA sequence which targets the I-*Ppo*I intron 3 sequence at position 345bp – 369bp. It also carries the I-*Ppo*I cleavage site and the ribozyme expression is under the control of the constitutive OmpA promoter. Transcription of the ribozyme is terminated at the T7 terminator. The entire gene cassette was synthesised with oligonucleotides as described in "Methods". The resulting plasmid pRBS 43 was transformed into the host strain JM109 (DE3) and tested for stability and self-restriction. Upon induction with 1 mM IPTG for 1 hour some plasmid is lost (Fig. 4). Of particular significance is that in the uninduced state there was a large amount of plasmid present which contrasts with that seen with the vector pRBS 22. This data indicates that the presence of the ribozyme does stabilise the plasmid, reducing the level of expression of the I-*Ppo*I enzyme in the uninduced state.

Two plausible reasons for the limited self-restriction of the plasmid upon induction may be:

(1) The I-*Ppo*I restriction site is adjacent to the OmpA promoter and hence the constitutive mRNA transcription from the promoter may sterically hinder the I-*Ppo*I restriction enzyme from binding to the cleavage site.

(2) The OmpA promoter being a medium-level and constitutive promoter may result in excessive levels of the ribozyme which may result in minimal quantities of I-*Ppo*I mRNA that could be translated into active enzyme.

To test the above two hypotheses, two different plasmids were constructed. To test hypothesis (1), an additional I-*Ppo*I restriction site was cloned upstream of the T7 promoter in plasmid pRBS 43 to give plasmid pRBS 45 (Fig. 1) to determine if p*Omp*A-distal location of the I-*Ppo*I site may

enhance I-*Ppo*I binding and cleavage. To accomplish this, the plasmid pRBS 43 was digested with *Ssp* I, CIP treated and ligated to the I-*Ppo*I site fragment generated by annealing and cleavage with *Sma* I of oligos RBS 3 and RBS 4 (Table 1). This resulted in plasmid pRBS 45.

For hypothesis (2), the OmpA promoter in plasmid pRBS 43 was replaced with a part of the iron regulated aerobactin promoter designated p aer (Bindereif and Neilands, 1985). This promoter is normally expressed at basal levels and is induced to higher expression under iron limiting conditions. *In-vitro*, such conditions are achieved by the addition of 200uM 2',2-Dipyridyl (iron chelator) to the growth media. This plasmid was designated pRBS 46 (Fig. 1). The cloning was carried out by PCR amplifying p*aer* from plasmid pHB170 with oligos RBS 87 and RBS 88 (Table 1). cleavage with *Bgl* II and *Xho* I and cloning the fragment into the respective sites of plasmid pRBS 43.

Both plasmids were transformed into host strain JM109 (DE3) and examined for plasmid maintenance under uninduced conditions and self-digestion following induction of I-*Ppo*I expression.

Initial experiments showed that plasmid pRBS 45 was rapidly digested even in the uninduced state suggesting that either the distal location or the additional I-*Ppo*I site on the plasmid results in rapid plasmid cleavage. It has been previously established that ribozymes are more active at higher temperature and our previous experiments had established that I-*Ppo*I activity is reduced at 42°C. An experiment was therefore conducted to determine if the plasmid could be maintained in the cell under uninduced conditions due to the partial inactivation of I-*Ppo*I and greater activity of the ribozyme. The recombinant cultures were grown initially at either 37°C or 42°C and uninduced and induced (for I-*Ppo*I expression) plasmid profiles were analysed as shown in Fig. 5. The data revealed that at 42°C, the recombinant plasmid was maintained in the cell presumably due to the combined effects of partial inactivation of I-*Ppo*I and greater activity of the ribozyme at the higher temperature. Upon induction at 37°C, a significant amount of plasmid is self-restricted. Although the data is interesting, it does not allow a practical application for the suicide plasmid in bacterial strains either for vaccines or bioremediation. The growth at 42°C is also unsuitable since prolonged growth at such higher temperatures may be detrimental for the survival of the carrier micro-organisms. For the development of strains

for bioremediation, the higher temperature may also adversely affect the activity of the enzymes used to target the toxic waste.

Plasmid cleavage of pRBS 46 (Fig. 6) was similar to that of pRBS 43 (Fig. 2) suggesting that although the p*aer* promoter (pRBS 46) can be repressed unlike the p*OmpA* (pRBS 43), both promoters express sufficient ribozyme to inhibit plasmid cleavage.

There are at least two plausible reasons for the above observations:

(1) Upstream of p*OmpA* (pRBS 43) and paer (pRBS 46), is located the strong promoter p*lac*. It may be possible that this promoter may override p*OmpA* or p*aer* thereby expressing more ribozyme.

(2) Although p*OmpA* is replaced with p*aer* in plasmid pRBS 46, the plasmid does not carry the distal I-*Ppo*I site and hence it is still possible that the p*aer*mRNA transcript may sterically hinder the binding of I-*Ppo*I to the promoter proximal I-*Ppo*I site.

To address the above two possibilities, an additional two plasmids were constructed namely pRBS 47 and pRBS 48 (Fig. 1).

To eliminate the potential over-riding effect of p*lac*, the *Xba* I / *Xho* I insert DNA in pRBS 46 was cloned into the respective sites of plasmid pET-21d to give plasmid pRBS 47. This plasmid does not carry additional promoters upstream of the *Xho* I site and hence the ribozyme should be expressed solely under p*aer*. Six independent isolates were grown and plasmid profiles from uninduced and induced (1mM IPTG for 1 hr.) recombinant cells were analysed by agarose electrophoresis (Fig. 7). The results demonstrated that the plasmid was well maintained in the cells and there was no evidence of self-restriction. This data along with that observed (described above) for plasmids pRBS 43 and pRBS 46 indicates that the strength of the promoter expressing the ribozyme is not a critical factor in achieving plasmid self-restriction.

To test hypothesis (2) described above, an additional I-*Ppo*I restriction site was cloned distal to the promoter-proximal site. This was achieved by annealing oligonucleotides RBS 3 and RBS 4 (Table 1) (generates an I-*Ppo*I site), cleaving with *Sma* I and cloning it into the unique *Psh*A1 (Fig. 1) site of plasmid pRBS 47 to result in plasmid pRBS 48 (Fig. 1). Six independent isolates were grown and plasmid profiles from uninduced and induced (1mM IPTG for 1 hr.) recombinant cells were analysed by agarose electrophoresis (Fig. 8). The results clearly demonstrate effective plasmid maintenance in

uninduced cells and almost complete plasmid self-restriction upon induction of I-*Ppo*I expression.

The above data establishes the viability and utility of the suicide expression vector of the present invention, by showing that:

(1) The eucaryotic restriction enzyme I-*Ppo*I does not cleave *E. coli* and *Salmonella typhimurium* chromosomes hence such an enzyme if expressed in the bacterial cytoplasm will not be deleterious to the viability of the bacterial cell.

(2) I-*Ppo*I can be expressed in the bacterial cytoplasm using either the T7 promoter or ideally any other repressible bacterial promoter.

(3) I-*Ppo*I is active in the procaryotic bacterial cell and is able to cleave recombinant plasmid harboured I-*Ppo*I restriction site(s).

(4) Once I-*Ppo*I cleaves the recombinant plasmid endogenous bacterial nucleases rapidly eliminate the plasmid DNA.

(5) Since most bacterial promoters are "leaky" for expression, the ribozyme technology can be used to cleave I-*Ppo*I mRNA under non-induced conditions. This property is useful for plasmid maintenance in the cell during the process of plasmid-borne recombinant foreign antigen expression.

(6) The ribozyme can be expressed using an inducible promoter like *paer* since it may be possible to modulate the amount of ribozyme produced.

(7) The I-*Ppo*I site should ideally be located at a site distal to any promoter since promoter transcription may sterically hinder I-*Ppo*I binding to the recognition site.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

References:-

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